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Award Number: DAMD17-01-1-0793

TITLE: Disruption of Dopaminergic and Cholinergic Function in Military Deployment: Implications to Parkinson's Disease

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REPORT DATE: September 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2003	Annual (15 Aug 02-14 Aug 03)

4. TITLE AND SUBTITLE
Disruption of Dopaminergic and Cholinergic Function in Military Deployment: Implications to Parkinson's Disease

5. FUNDING NUMBERS
DAMD17-01-1-0793

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The goal of this project is to evaluate the potential of pesticides and other compounds used by the military for their potential to damage the brain dopamine system and increase the risk for Parkinson's disease. Notable research accomplishments over the past year include the following: Deltamethrin increased the expression of DAT, TH, and VMAT2, locomotor activity in C57BL mice. Deltamethrin did not exacerbate the toxicity to the dopamine neurotoxin MPTP either given before or after MPTP. Chlorpyrifos had no effect on dopamine uptake in neuroblastoma cells and did not exacerbate MPTP toxicity. Pyridostigmine bromide had no effect on dopamine uptake in neuroblastoma cells. JP-8 jet fuel is toxic to neuroblastoma cells only at 1 mM concentrations. No toxicity was seen at concentrations from 100 nM to 500 μM. Thus, with all of the compounds studied we have not observed toxicity consistent with a compound that would be thought to cause overt damage to the dopamine system. However, we have seen alterations of the dopamine system that must be studied further. The completion of this study will reveal the impact of militarily relevant agents on the pathogenesis of Parkinson's disease and hopefully lead to strategies and policies that reduce the incidence of the disease.

14. SUBJECT TERMS
pyrethroid, acetylcholinesterase inhibition, deltamethrin, pyridostigmine bromide, microarrays

15. NUMBER OF PAGES
12

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

This report summarizes the key findings of Year 2 of this project. The following Statement of Work is excerpted from the original project, but has been modified to include the supplement on JP-8 jet fuel.

Statement of Work

Specific Aim 1. Effects of pyrethroids, acetylcholinesterase inhibitors, and JP-8 jet fuel on dopamine uptake, DAT localization, and MPP^+ induced apoptosis in DAT expressing cells. This aim will test the hypothesis that pyrethroids and acetylcholinesterase inhibitors increase MPP^+-induced apoptosis primarily through acting on dopamine uptake. The following experiments will be performed under this aim:

- **I)** Perform dopamine uptake tests on DAT expressing cells treated with deltamethrin, permethrin, chlorpyrifos, pyridostigmine bromide, and MPP^+. Years 1-2
- **II)** Determine the effects of deltamethrin, permethrin, chlorpyrifos, pyridostigmine bromide, JP-8, and MPP^+ on DAT localization in DAT expressing cells. Year 3-4
- **III)** Perform caspase 3 assays on cells treated with deltamethrin, permethrin, chlorpyrifos, JP-8, and pyridostigmine bromide to determine if they cause apoptosis or exacerbate MPP^+-mediated apoptosis. Year 1-2

Specific Aim 2. Examine the effects of pyrethroids, acetylcholinesterase inhibitors, JP-8, and MPTP on mouse behavior and dopaminergic and cholinergic gene and protein expression. This aim will test the hypothesis that the combination of pyrethroids and acetylcholinesterase inhibitors decreases dopaminergic activity and increases cholinergic activity, resulting in impaired locomotion in C57BL/6 mice. *An important feature of this aim is that we will examine behavior, gene expression, protein expression, and neurotransmitter levels in the same animals.*

Aim 2A. Assess effects of pyrethroids, acetylcholinesterase inhibitors, and their combination on mouse behavior. In addition, JP-8 will also be tested. This aim will test the hypothesis that these compounds decrease locomotion and increase anxiety and aggression.

- **IV)** Perform locomotor activity, open field ambulation, elevated plus maze, and social interaction tests on C57BL mice six days after MPTP treatment. 3 days prior to or 3 days following MPTP treatment, mice will be treated with 9 mg/kg of deltamethrin, chlorpyrifos, neostigmine, or the combination of deltamethrin and chlorpyrifos. Year 1, 2

Aim 2B. Immunochemical and neurochemical analysis of dopaminergic and cholinergic systems following pyrethroids, acetylcholinesterase inhibitors, JP-8, and MPTP. This aim will assess the effects of pyrethroids, acetylcholinesterase inhibitors, and MPTP on cholinergic and dopaminergic protein expression and function.

- **V)** On the same mice in Aim 2A perform immunoblotting for DAT, D1, tyrosine hydroxylase, M1 and M2 receptors, vesicular acetylcholine transporter, and choline acetyltransferase. Year 1.5 to 2.5
- **VI)** Perform HPLC analysis of monoamines on mice from Aim 2A. Year 2
- **VII)** On a separate subset of animals treated with deltamethrin, chlorpyrifos, neostigmine, and MPTP, perform striatal dopamine and choline uptake. Year 3.

Aim 2C. Use custom cDNA microarrays to analyze regional changes in dopaminergic and cholinergic gene expression following pyrethroids, acetylcholinesterase inhibitors, JP-8, and MPTP. VIII) We will perform cDNA microarray analysis on midbrain, basal forebrain, and striatum from ice treated with deltamethrin, chlorpyrifos, or MPTP. Years 2 and 3 will contain most of the actual hybridizations. Year 4 will be focused bioinformatic analysis. Years 2,3,4

General Summary

Dr. Miller's lab has successfully completed the transition to Emory University. His laboratory is now fully functional and he has hired the appropriate staff for this project. Scott Mordecai, who used to work as an applications specialist for Affymetrix has joined Dr. Miller's staff. He is responsible for the molecular analyses in these studies. Dr. Miller was also contacted by DOD staff regarding testing the effects of JP8 jet fuel in our system. This supplement was finally approved and funded in September, which is actually the beginning of year 3 (and will be funded for 3 years), but we do already have some preliminary data on the toxicity of JP8 in neuroblastoma cells.

During a summer rotation, a neuroscience graduate student made a very interesting observation with the pyrethroid insecticides being studied in this project. He demonstrated that exposure to deltamethrin appears to increase the sensitivity of mice to the effects of cocaine. This was based upon a parallel hypothesis that the increase in DAT we suspect my increase vulnerability to dopamine cell death may also alter response to drugs of abuse that target the dopamine system. Most notable was that this rotation project has led to Thomas Guillot selecting Dr. Miller's lab for his dissertation project so he is now part of this project.

The key investigators for this project are Dr. Miller, Mr. Scott Mordecai, Dr. Min Wang, Mr. Thomas Guillot, and Dr. Jason Richardson. Dr. Elwan, who had previously worked on this project has returned to Egypt this past year. His responsibilities have been assumed by Thomas Guillot under Jason Richardson's guidance. Dr. Min Wang has been assisting with the behavioral studies. Scott Mordecai is responsible for performing the cDNA microarray analysis.

Scientific Progress

We have made significant progress in our Statement of Work. As noted starting in September 2003 we were awarded a supplement to examine JP-8 in our experimental models. Figure 1 shows initial toxicity assays with JP-8. Surprisingly, JP-8 was only toxic to neuroblastoma cells at very high concentrations (1 mM). We are now starting the studies on other outcomes (uptake and trafficking).

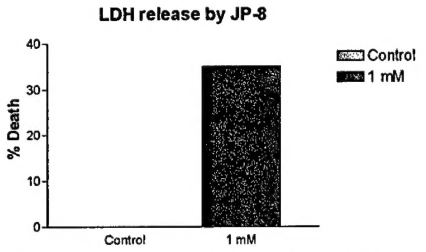


Figure 1. JP-8 was incubated with SK-N-MC cells for 24 hours. Dosages tested were 1 mM, 500 µM, 250 µM, 100 µM, 50 µM, 25 µM, 10 µM, 1 µM, and 100 nM. 1 mM was the only concentration that elicited cell death significantly greater than control.

In Specific Aim 1, we proposed to determine the effects of pyrethroids and acetylcholinesterase inhibitors. Much of these data were submitted with the previous progress report for Year 1 of the project. In Year 2, we have extended the observations from Year 1 in which we demonstrated that deltamethrin and permethrin only reduced DAT-mediated dopamine uptake after a 24 hour exposure by performing full kinetic analyses of DAT-mediated dopamine uptake to determine whether this was a direct effect on DAT. In Figure 2, we demonstrate that there is a selective decrease in the Vmax of DAT 24 hours after exposure to permethrin or deltamethrin, suggesting that these compounds do not competitively inhibit DAT and that there are other mechanisms responsible. Previously we had reported that there was no increased lactate dehydrogenase (LDH) activity elicited by these compounds at this time point, indicating no overt cell death. Therefore, we determined the levels of DAT in the cells by western blot and determined that there was no specific loss of DAT protein (data not shown). Thus, we concluded that there must be an alternate explanation for this decrease in uptake and that it may be related to malfunctioning of the cell itself. Since LDH is only indicative of a breach in the plasma membrane of the cell, we performed an oligonucleosome ELISA, which measures apoptosis by determining cytoplasmic levels of free oligonucleosomes indicative of DNA fragmentation, to determine whether induction of apoptosis was responsible for the decrease in uptake. Figure 3 shows that after 24 hr of exposure, both deltamethrin and permethrin induce apoptosis, which corresponds to the same time point at which we observed significant reductions in dopamine uptake. Also, note that permethrin was much more potent at inducing apoptosis than deltamethrin. These data

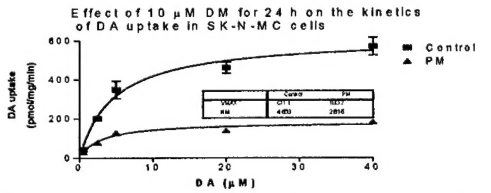


Figure 2. Deltamethrin noncompetitively inhibits DAT-mediated dopamine uptake in a non-competetive manner after 24 hr of exposure as evidenced by a

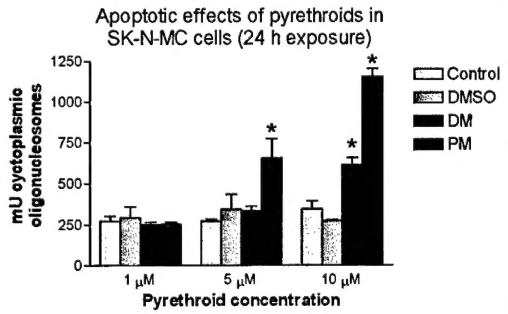


Figure 3. Permethrin and deltamethrin induce apoptosis in a dose-related manner with a similar time course as seen with reduction of dopamine uptake

suggest that apoptosis may play a role in the reduction of dopamine uptake observed at 24 hrs. All of these data were part of a poster presented by Dr. Elwan at the FASEB meeting this year (Elwan et al., 2003), and a manuscript of these data is currently in preparation. In addition to the data presented above, we are now in the progress of determining caspase activity at this same time point. Another possible explanation for these reductions is reduction of DAT expression on the plasma membrane. As mentioned previously, the total levels of DAT were not reduced at this time point. However, DAT must be present on the plasma membrane in order to function properly. We are now in the process of determining the amount of DAT present on the membrane by cell-surface biotinylation. We have also determined the effects of pyridostigmine bromide and chlorpyrifos on DAT-mediated dopamine uptake. Similar to the pyrethroids, there was no effect on dopamine uptake determined at 10 or 30 min with concentrations of up to 10 μM of chlorpyrifos or pyridostigmine bromide. In contrast to the pyrethroids, neither had an effect on dopamine uptake when incubated for 24 hrs (data not shown). We have previously reported on the effects of MPP+ on DAT function, demonstrating the inhibitory potency of MPP+ for DAT and its potential for initiating apoptosis by determining caspase 3 activity and free oligonucleosome concentrations. Thus, it appears that deltamethrin and permethrin have no direct effect on DAT function *in vitro* and instead appear to elicit toxicity through an apoptotic mechanism. These findings have important implications on the mechanism of DAT upregulation by repeated exposure to pyrethroids, which we demonstrate below. These data suggest that direct interaction of pyrethroids and the transporter are unlikely, and that upregulation occurs through another mechanism, possibly involving the trafficking or transcriptional regulation of DAT. The assays that we have proposed for DAT trafficking and the microarray data should aid us in determining this mechanism.

In Specific Aim 2, we proposed to examine the effects of pyrethroids, acetylcholinesterase inhibitors, and their combinations on mouse behavior and dopaminergic and cholinergic gene and protein expression. We proposed two experimental paradigms for determining the effects of these compounds in mice. First, we exposed animals to the dopaminergic neurotoxin MPTP followed by exposure to deltamethrin, chlorpyrifos, neostigmine, or the combination of chlorpyrifos and deltamethrin. Instead of using the pharmacological agent neostigmine, to which there is little possibility for military personnel to be exposed, we decided to utilize pyridostigmine bromide, which has the same mechanism of

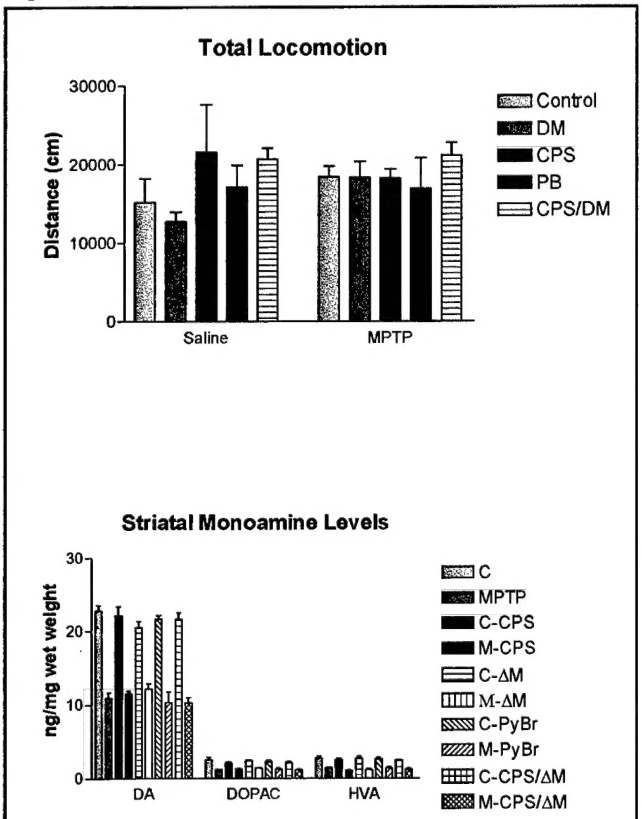
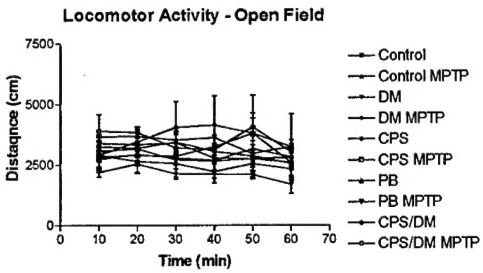


Figure 4. Effects of exposure to 2 x 10 mg/kg MPTP (M) or Saline (C)combined dosing of MPTP and chlorpyrifos (CPS), deltamethrin (ΔM), or pyridostigmine bromide (Py Br) on locomotor activity and striatal neurochemistry.

action as neostigmine, but is more relevant to military personnel. Figure 4 shows the effects of deltamethrin, chlorpyrifos, pyridostigmine bromide, MPTP, and their combinations on locomotor activity in mice. When we examined total locomotor activity among the treatment groups, we found little differences. To examine the effects of these toxicants and MPTP on dopaminergic chemistry, we determined the levels of dopamine and its metabolites, DOPAC and HVA, in the striatum of mice which received the toxicant alone, or in combination with MPTP (2x10 mg/kg). MPTP alone significantly decreased the levels of dopamine and its metabolites by 50-60% (Figure 4). None of the other tested compounds significantly affected dopaminergic neurochemistry and did not exacerbate MPTP toxicity. The HPLC data were confirmed by western blots of DAT, the vesicular monoamine transporter 2, and tyrosine hydroxylase (data not shown). We are currently in the process of determining the effects on cholinergic neurochemistry.



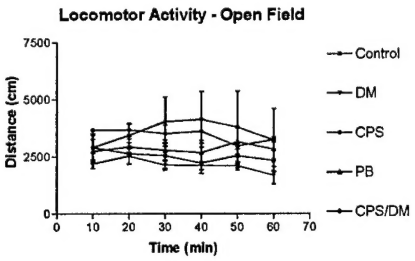
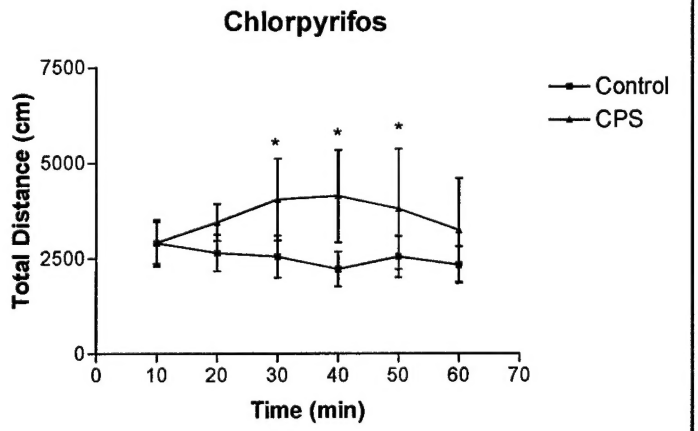


Figure 5. Effects of deltamethrin (DM), chlorpyrifos (CPS) on locomotor activity in mice. Top panel, individual and combined doses were given. Bottom panel, graph showing only the individual compounds.

As mentioned above, we found no differences in total locomotor activity between the various treatment groups. However, when we analyzed activity in 10 min blocks over the total 60 min testing period, we found significant hyperactivity induced by chlorpyrifos alone (Figures 5 and 6). In addition, MPTP appeared to potentiate the increased locomotor activity of the deltamethrin treated group (Figures 5 and 6). MPTP caused no reduction of activity in the open-field. It is not surprising that MPTP did not show an effect in this assay. This is why we have been developing new assays to assess locomotor activity. We are still in the process of analyzing data from our other behavioral assays.



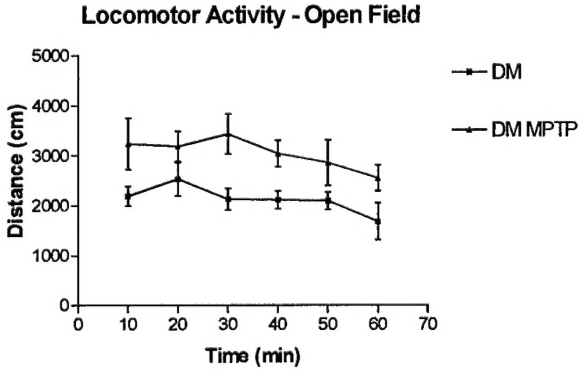


Figure 6. Chlorpyrifos (CPS) causes hyperactivity in the open-field. MPTP enhances the hyperactive effects of deltamethrin (DM).

In the second exposure paradigm, MPTP was given first followed by the same toxicants used above. Similar to the previous study, we found that there were no significant alterations in total locomotor activity and no differences in striatal monoamine levels measured by HPLC (Figure 7). The lack of exacerbation of MPTP toxicity based on the HPLC data was confirmed by western blots as in the previous exposure paradigm (data not shown).

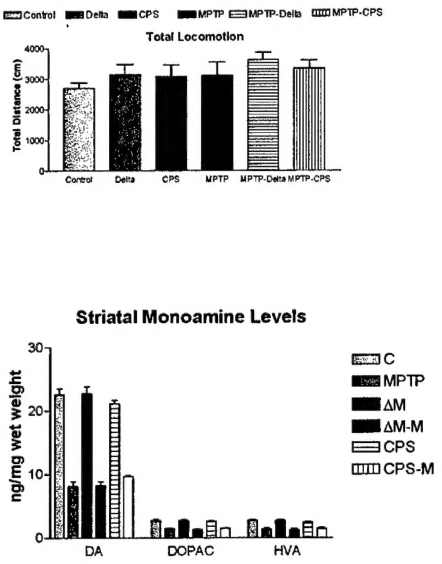


Figure 7. No significant effects on total locomotor activity was detected in animals exposed to deltamethrin (ΔM), chlorpyrifos (CPS), or the combination of the two. MPTP elicited a significant decrease (50-60%) in striatal dopamine, which was not exacerbated by previous pesticide exposure.

Because we saw no effect on DAT expression with a single exposure of deltamethrin, which is similar to that observed in the cell culture studies in Specific Aim 1, we utilized a 3 dose paradigm with which we have previously observed upregulation of DAT to explore the effects of deltamethrin on the dopaminergic system. Mice were exposed to deltamethrin (6 mg/kg ip on days 1, 8, and 15). This study confirmed our previous observation of deltamethrin upregulation of DAT, as measured by western immunoblotting and 3H-WIN 35,428 radioligand binding (Figure 8). In addition, we determined that deltamethrin induced hyperactivity in the open field (Figure 9).

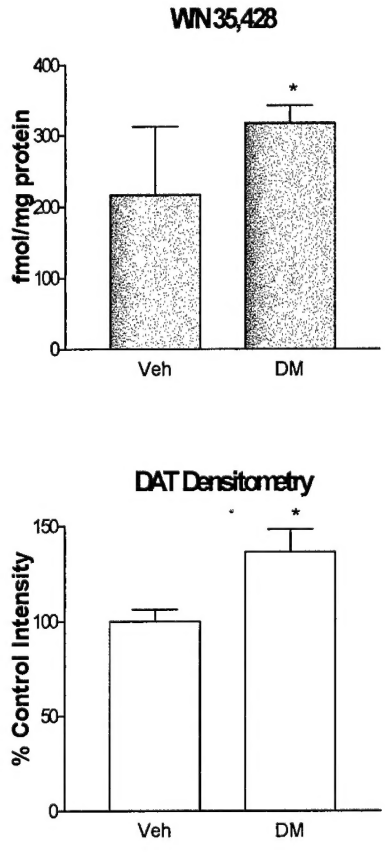


Figure 8. Deltamethrin increases the expression of DAT as measured by radioligand binding and by western blotting.

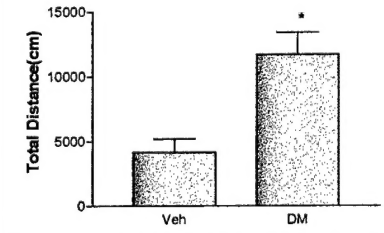


Figure 9. Effects of deltamethrin (6 mg/kg ip on days 1, 8, and 15) on locomotor activity. Deltamethrin significantly induces hyperactivity in C57BL mice.

We also determined that this upregulation of DAT enhanced the psychostimulant effects

of cocaine by potentiating the hyperactivity induced by cocaine. As mentioned above, this was part of Mr. Thomas Guillot's summer rotation project, from which he ultimately decided to join Dr. Miller's lab and continue researching the impact of pyrethroid modulation on the dopaminergic system. While not a part of the proposed project, this demonstrates the potential of pesticide modulation of the dopaminergic system exacerbating the effects of other agents that target the nervous system. In addition to the upregulation of DAT, we observed that the vesicular monoamine transporter 2, tyrosine hydroxylase, and phosphorylated tyrosine hydroxylase were all upregulated by repeated exposure to deltamethrin (Figure 10). Taken in concert these data suggest that deltamethrin targets multiple components of the dopaminergic system which may render it more vulnerable to future toxic insults. We are in the process of examining the effects of MPTP on mice exposed repeatedly to deltamethrin.

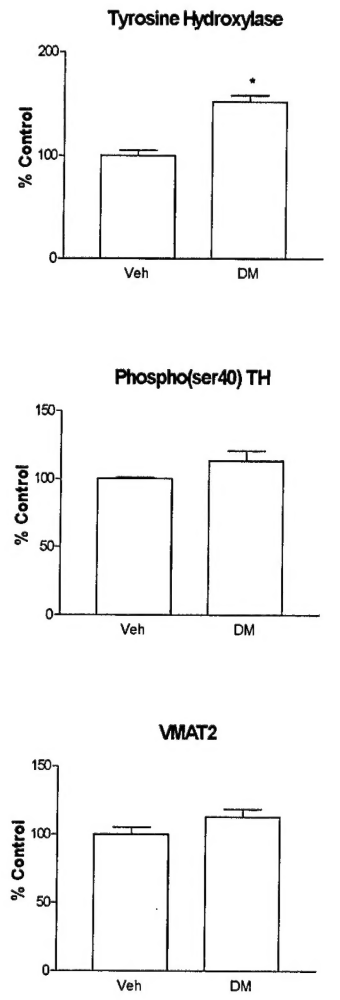


Figure 10. In order to address the deltamethrin induced hyperactivity, we examined the effects of deltamethrin on tyrosine hydroxylase and VMAT2. It appears that deltamethrin treatment upregulates the dopamine system.

Microarray studies. As mentioned above Dr. Miller has hired Scott Mordecai to perform these studies. Mr. Mordecai has extensive experience with the Affymetrix system, has established the mRNA isolation procedures, and is in the process of running test arrays. We should begin generating array data by the end of 2003.

Conclusions

Excellent progress has been made on this project in the past year. We have completed almost all of the in vitro studies and have gained valuable insight into the potential mechanisms by which pyrethroids alter dopaminergic neurochemistry. In addition, significant progress has been made with the in vivo studies. In this report, we show data that suggest that acute exposure to pyrethroids does not appear to alter dopaminergic neurochemistry, but rather repeated exposure to lower levels of deltamethrin have a significant impact on the dopaminergic system and may render it more susceptible to toxic insult. This is an important finding with reference to the deployed soldier who is more likely to encounter to multiple lower-level exposures rather than one single exposure. The future studies proposed in the initial grant submission will give us valuable insight into the mechanism of pyrethroid action on the dopamine system and how these exposures may be involved with Parkinson's disease. In addition, we hope that these data will ultimately help to better protect military personnel both at home and abroad.

References
None

Appendix
Copy of poster presented at the Federation of American Societies for Experimental Biology

Key Research Accomplishments for Year 2
Repeated exposure to deltamethrin increases the expression of DAT in C57BL mice
Repeated exposure to deltamethrin increases locomotor activity in C57BL mice
Repeated exposure to deltamethrin increase the expression of the dopamine transporter, tyrosine hydroxylase, and the vesicular monoamine transporter
Acute deltamethrin pretreatment does not exacerbate MPTP toxicity
Acute exposure to chlorpyrifos does not exacerbate MPTP toxicity
Acute deltamethrin treatment after MPTP exposure does not exacerbate toxicity

Pyridostigmine bromide has no effect on dopamine uptake in neuroblastoma cells
Chlorpyrifos has no effect on dopamine uptake in neuroblastoma cells

JP-8 jet fuel is toxic to neuroblastoma cells only at 1 mM concentrations. No toxicity was seen at concentrations from 100 nM to 500 μM

Reportable outcomes
Poster presented at FASEB meeting

Elwan, M.A., Caudle, W.M., Richardson, J.R., and Miller, G.W. (2003). Effect of Pyrethroids on Dopamine Uptake in SK-N-MC Cells Expressing the Dopamine Transporter. FASEB J. 17:6670.

Appendix

Effect of pyrethroids on dopamine uptake in SK-N-MC cells expressing the dopamine transporter

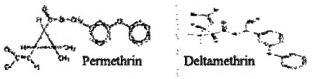
Mohamed A. Elwan, William M Caudle, Jason R Richardson and Gary W. Miller

Center for Neurodegenerative Disease and Department of Environmental and Occupational Health, Rollins School of Public Health, Emory University, Atlanta, GA.

ABSTRACT

Parkinson's disease (PD) is a progressive neurodegenerative disease affecting the nigrostriatal dopaminergic pathway. Several studies have demonstrated a strong correlation between exposure to insecticides and the incidence of PD. The dopamine transporter (DAT) plays a crucial role in the regulation of dopaminergic transmission and is thought to be the route through which environmental toxins gain access to dopaminergic neurons. In this study we examined the effects of two pyrethroids, permethrin (PM, type I) and deltamethrin (DM, type II) on dopamine (DA) uptake using human neuroblastoma SK-N-MC cells stably expressing human DAT. Cells were treated for 10 min, 30 min, or 24 h with various concentrations of either PM or DM and then tested for their ability to transport DA. Incubation with 10 μM of PM or DM (the highest concentration used) for 10 min produced no significant change in DA uptake, while 30 min incubation decreased DA uptake by approximately 20-25%. On the other hand, cells incubated for 24 h showed approximately 75% decrease of DA uptake in the presence of either pyrethroid at 5 or 10 μM. Incubation for 24 h with 1 μM PM or DM resulted in the inhibition of DA uptake by 32% and 42%, respectively. Our results suggest that both pyrethroids inhibit DA uptake in a time- and dose-dependent fashion. The mechanism by which pyrethroids decrease DA uptake remains to be elucidated. However, decreased DA uptake may explain, in part, the motor disorders observed in Gulf War Syndrome and that observed with pyrethroid toxicity.

Introduction



- Parkinson disease (PD) is a neurodegenerative disorder affecting the nigrostriatal dopaminergic neurons.

-Both genetic and environmental factors are thought to be involved in this neurodegeneration process.

- Among the environmental factors that might increase the incidence of PD is the exposure to pesticides.

-Numerous evidence indicate that exposure to pesticides through drinking well water, agricultural work and living in rural areas is associated with increased risk of PD.

-Dopamine transporter (DAT) plays a crucial role in regulating the level of extracellular dopamine and also is thought to be the route through which environmental toxins gain access to dopaminergic neurons.

- In this study we sought to study the effect of two pyrethroids (permethrin, type I, and deltamethrin, type II) on dopamine uptake using a cellular model (SK-N-MC cells expressing DAT).

Methods

Cell Culture

SK-N-MC cells stably expressing human DAT were maintained at 37°C, 5%CO_2, in MEM medium, supplemented with 10% fetal bovine serum, 50U/ml penicillin, 50μg/ml streptomycin, 2mM L-glutamine, 1mM sodium pyruvate and non-essential amino acids.

Dopamine Uptake

Dopamine uptake was determined as described by Pifl et al. (1993). Briefly, cells were incubated with either deltamethrin (DM) or permethrin (PM) for the specified times prior to addition of dopamine. Reactions were allowed to proceed for 5 min. Radioactivity was determined and normalized to the protein concentration. Nonspecific uptake was determined in the presence of GBR 12935.

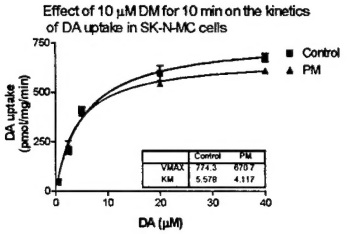
Cytotoxicity assay

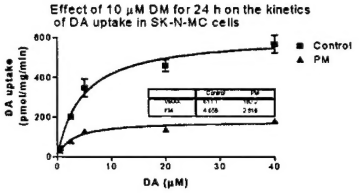
The possible cytotoxic effect of pyrethroids was measured as the lactate dehydrogenase (LDH) activity released in the cell incubation media using cytotoxicity detection kit (Roche).

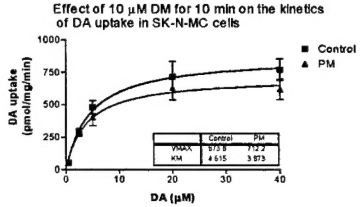
Apoptosis assay

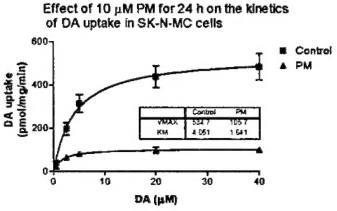
The possible apoptotic activity of pyrethroids was assayed by determining the levels of cytoplasmic mono- and oligonucleosomes in the lyaste of cells treated with either DM or PM. Cell death detection ELISA kit (Roche) was used.

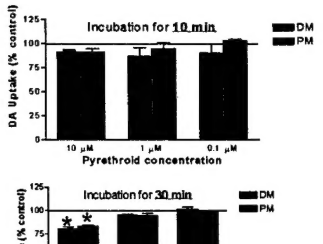
Results

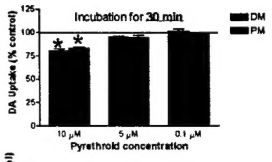


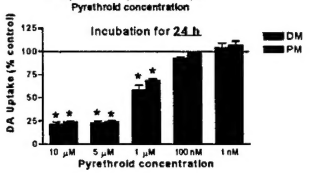


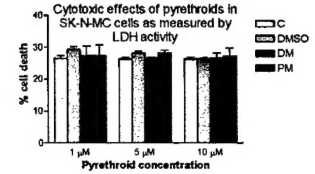


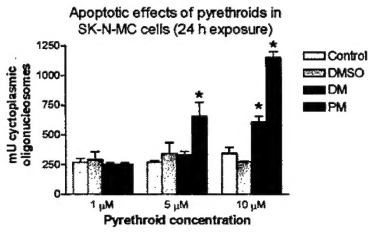












Conclusion

-Both DM and PM inhibited the dopamine uptake in SK-N-MC cells.

-This inhibition of dopamine uptake seems to be dependent on both the pyrethroid concentration used as well as on the exposure time.

- At 10 min incubation, both compounds are devoid of any significant DIRECT effect (increase or decrease) on dopamine uptake, indicating the absence of a cocaine like effect.

-At the highest concentration attainable (10 μM), neither DM nor PM produced any significant cytotoxic effect as revealed by LDH assay. This indicates that decreased dopamine uptake is not due to cytotoxicity.

-Exposure of cells to 10 μM of either DM or PM for 24 h significantly increased cell apoptosis, while at 5 μM concentration only PM produced appreciable cell apoptosis.

-Induction of cell apoptosis may, in part, explain the inhibition of dopamine uptake in cells exposed to pyrethroids and may shed some light on the possible mechanisms of motor disorder symptoms of pyrethroid toxicity in humans.

- Mitochondrial toxicity with decreased energy production, and DAT trafficking are among other mecahnisms that may explain decreased dopamine uptake, are currently under investigation.

References

Pifl C et al. (1993). J. Neurosci. 13:4246-4253.